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TITLE: Rescue of TET2 Haploinsufficiency in Myelodysplastic Syndrome Patients Using Turbo Cosubstrate

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14. ABSTRACT
We continued with our focus on the large scale expression of TET2 dioxygenase. During this period we developed a convenient assay for TET2 dioxygenase, which will be utilized to determine the kinetics of wild-type and various clinical mutants of TET2 dioxygenase. Further, we made a library of putative 2-oxoglutarate analogs which will be used to rescue the activity of TET2 mutants from MDS patient. Initial results shows that the activity of TET2 can be modified using 2-oxoglutarate analogs.

15. SUBJECT TERMS

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Table of Contents

1. **INTRODUCTION:** TET2 is one of the most frequently mutated genes in myelodysplastic syndromes (MDS). The TET2 mutations are also prevalent in a number of myeloid malignancies such as MDS-myeloproliferative neoplasms (MDS-MPN) and acute myeloid leukemia derived from MDS and MDS-MPN (sAML). One of the fundamental causes of these diseases is the presence of 5-methylcytosine (5mC) marks, particularly in the CpG islands of promoters, leading to gene silencing. The wild-type (wt)-TET2 protein, a putative tumor suppressor, is a non-heme iron(II), 2-oxoglutarate (2OG)-dependent dioxygenase which initiates 5mC demethylation by hydroxylating it into 5-hydroxymethylcytosine (5hmC). TET2-knockout mice, which are viable and grossly normal initially, with age, develop diverse myeloid malignancies similar to humans. The objective in this project is to develop effective strategies using 2OG analogs to enhance the activity of the wt-TET2 enzyme both in vitro and in vivo in order to overcome TET2 haploinsufficiency. We have developed large scale expression of TET2 dioxygenase and a convenient assay for TET2 dioxygenase, which will be utilized to determine the kinetics of wild-type and various clinical mutants of TET2 dioxygenase. Further, we made a library of putative 2-oxoglutarate analogs which will be used to rescue the activity of TET2 mutants from MDS patient. Initial results shows that the activity of TET2 can be modified using 2-oxoglutarate analogs.

2. **KEYWORDS:** Myelodysplastic syndromes (MDS), MDS-myeloproliferative neoplasms (MDS-MPN), Acute myeloid leukemia (AML), 5-methylcytosine (5mC), Mutation, Haploinsufficiency, Small molecule activators, TET2, Dioxygenase, 2-oxoglutarate (2OG).

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**

Task 1: Screen a library of 2OG analogs and identify cosubstrates with better K_M values towards wt-TET2 under *in vitro* assay conditions (months 1-26).

1a. Scale-up the purification of wt-TET2 from the insect cells (SF9) using an N-terminal his-tag by affinity chromatography (months 1-6). We already have a stock of P1 virus, which will be used to produce ≈10 mg of pure wt-TET2 dioxygenase.

100% completed. Along with expression in the insect cell lines, we have developed a convenience expression of active TET2 enzymes in bacterial system, which is described later.

1b. Determination of kinetic properties (V_{max}, K_M, and k_{cat}) of wt-TET2 with respect to 2OG using the standardized *in vitro* HPLC assay (months 4-6). Using our reported methods we will determine the kinetic properties of wt-TET2 dioxygenase.

50% completed. Delays in progress is due to development of a reliable assay. As describe below we have tried several assays and found mass spectrometry-based assay to be most reliable.

1c. Synthesize, purify and characterize a library of 2OG analogs using the scheme reported in the application (months 1-18).

50% completed.

1d. Determination of kinetic properties (V_{max}, K_M, and k_{cat}) of wt-TET2 with respect to 2OG analogs using the *in vitro* HPLC assay (months 6-24). This will be followed by selection of ≈10 best 2OG analogs with an improved K_M value.

Ongoing. ***Our initial results shows for the first time that the activity of TET2 can be modulated, even enhanced in case of some MDS mutants, using 2-oxoglutarate analogs.*** We are continuing on this extremely exciting results and hope to publish some very significant papers from this grant.

1e. The shortlisted 2OG analogs (≈10) will be assayed with histone lysine demethylases, HIF prolyl hydroxylases and AlkB2 dioxygenases to identify 2OG analogs that show specificity towards wt-TET2 activation compared to other dioxygenases (months 12-24). From these experiments two 2OG analogs will be selected for the cell-based studies.

Not started yet.

Task 2: Develop strategies to improve wt-TET2 activity in haploinsufficient lymphoid cells from MDS patients (months 9-36).

2a. Selection at least two TET2 mutated haploinsufficient patient cell line and one normal cell line with wt-TET2, used as a control (months 12-18).

100% completed.

2b. Chemical modification (esterification) of the two 2OG analog selected from task 1e (months 24-26) and 2OG, which will be used as a control in every experiment.

Not started yet.

2c. Co-culture of the two TET2 mutated haploinsufficient patient cell line from task 2a in the presence or absence of the two modified 2OG analogs at five times K_M concentration. As a control, a normal cell line from healthy donor with wt-TET2 will be grown without any 2OG analogs (months 26-28).

Not started yet.

2d. Quantitation and analysis of 5mC/5hmC and gene expression levels in the three cell lines cultured in the presence or absence of modified 2OG analogs (months 28-36).

Not started yet.

- **What was accomplished under these goals?**

In Year 2 (June, 2014 – May, 2015) we continued with our focus on the large scale expression of TET2 dioxygenase. During this period we developed a convenient assay for TET2 dioxygenase, which will be utilized to determine the kinetics of wild-tye and various clinical mutants of TET2 dioxygenase. Further, we made a library of putative 2-oxoglutarate analogs which will be used to rescue the activity of TET2 mutants from MDS patient. The results of our studies are summarized below:

Large-scale expression of GST-TET2, His-TET2, and untagged TET2 dioxygenase: For *in vitro* assays a *TET2* fragment containing the dioxygenase domain along with the cystene-rich domain (encoding for 1129-2002 amino acids) was amplified by PCR Since the C-terminal region of the full-length TET2 harbors the cystene-rich and dioxygenase/hydroxylase domains, for *in vitro* assays, a *TET2* fragment containing the dioxygenase domain along with the cystene-rich domain (encoding for 1129-1936 amino acids with residues 1581-1843 replaced by a 15residue GS linker) was amplified by PCR. The amplified DNA sequence was gel purified and cloned into pGEM-T vector (Promega, Madison, WI). The sequences of TET2 gene was verified by DNA sequencing using SP6 and T7 promoter primers. The verified gene was subcloned into bacterial vectors for expression of TET2 with GST-tag, His-tag, and untagged proteins. The recombinant vectors were transformed into *Escherichia coli* BL21(DE3) cells for protein expression.

Purification of GST-TET2 and His-TET2dioxygenase: Cells were grown in Luria–Bertani broth containing 100 μg/ml ampicillin at 37 °C, until the OD_{600} of the culture reached 0.8. The optimized condition for recombinant protein expression was found to be 0.25-1 mM IPTG induction of bacterial culture (OD_{600} = 0.8) for 20 h at 20 °C. TET2 proteins (GST-TET2, His-TET2, and untagged TET2) were produced at ~5-10% of the total soluble protein observed using 10% SDS-PAGE analyses. The recombinant GST-TET2 protein was purified as described in the yr 1 report, while the recombinant His-TET2 protein, which is not yet reported in the literature, was purified using Ni-NTA column using standard procedures (Figure 1). In addition, we have expressed untagged TET2 protein in bacteria for the first time in literature and in the process of developing a novel ion-exchange purification method.

In vitro assays for the TET2 dioxygenase activity: We have developed three independent assays for the TET2 dioxygenase, which will be used to determine its kinetics using 2OG analogs.

1. Colorimetric TET Activity Assay: As described in year 1 report, we have also used a convenient 5mC-Hydroxylase TET Activity/Inhibition Assay Kit from Epigentek. In this assay, a methylated substrate (described above) is stably coated onto the microplate wells. Active TET2 dioxygenase converts the methylated substrate to hydroxymethylated product. The TET-converted hydroxymethylated products can be recognized with a specific antibody. The ratio or amount of hydroxymethylated products, which is proportional to enzyme activity, can then be colorimetrically measured by reading the absorbance in a microplate spectrophotometer at a wavelength of 450 nm. The activity of the TET2 enzyme is in turn proportional to the optical density intensity measured (Figure 2).

2. LCMS assay: After some delays, we have successfully developed a LCMS assay to determine the direct activity of TET2 dioxygenase. In this assay, double stranded DNA oligo nucleotides (containing one, two or more 5mC/s) were obtained from IDT. To quantify the substrate and product in TET2 catalyzed reaction, double stranded DNA oligo nucleotides were column purified. To the purified mixture, resuspended in 10 µL, 10 units of DNAse I (Qiagen) was added and incubated at 37°C for 12 hours. The DNAse I can utilize both single and double stranded DNA as substrate and produce 5′-phospho-mononucleotide and -oligonucleotide products. Samples from DNAse I treatment were subjected to calf intestinal alkaline phosphatase (CIAP from NEB) treatment using 4 units of CIAP for another 12 hours. The processed nucleosides were re-suspended and analyzed using a LCMS system.

The nucleosides were separated on a Phenomenex Gemini 5u C18 column at 0.5 ml/minute flow rate using a gradient method comprising solvent-A (10mM ammonium acetate, pH 4.0) and solvent-B (100% acetonitrile containing 0.1% acetic acid). Initially, we used phosphate buffer in solvents, which resulted in the desired separation of nucleosides. However, the residual phosphate ions suppressed the ionization of nucleosides during both the later LCMS and MALDI-TOF experiments. To overcome this problem, we shifted to ammonium acetate buffer system since this is a volatile system and can be efficiently removed during speed vac. The time profile of the gradient used in the ammonium acetate buffer is as follows: 100% solvent-A for the first 3 minutes followed by a gradient to reach 5% solvent-B in the next 20 minutes. This was followed by holding at 5% solvent-B for 3 minutes and then dropping to 0% solvent-B (100% solvent-A) in the next 2 minutes. The nucleosides were detected by UV at 280 nm. Using this method we were able to separate and detect, based on the elution of purified standards, all the nucleosides on the LCMS system (Figures 3). Our assay allows separation and quantification of the TET2 substrate and products, thus enabling characterization of the kinetics of TET2 dioxygenase.

3. Dot Blot assay: We have developed a novel dot blot assay to measure the activity of TET2 dioxygenase. In this in vitro assay, in contrast to in situ cellular dot blot using genomic DNA, we have used double stranded DNA oligo nucleotides containing one, two, or more 5mC/s as substrate (Figure 4). This convenient assay will be used as primary assay to screen 2OG analogs to rescue the activity of TET2 doixygenase.

Library of 2-oxoglutarate (2OG) analogs: Four clinically observed TET2 mutations at R1896 and S1898 residues have been made. To rescue the activity of these clinical mutations a library of 2OG analogs with a carbon chain length of 2-6 (similar to four carbons in 2OG) has been

made (Figure 5). Using this library, attempts will be made to rescue the activity of TET2 dioxygenase where the in vitro dot bot will be used and primary assay, while the LCMS and colorimetric assays will be used as secondary and tertiary assays. The active compounds will be analyzed with MDS patient cell lines.

Screening of 2OG analogs using mass spectrometry-based TET2 assay: We have started screening 2OG analogs to modulate the activity of the wt- and mutant-TET2 enzymes using LCMS assay described earlier. Our initial results shows for the first time that the activity of TET2 can be modulated, even enhanced in case of some MDS mutants, using 2-oxoglutarate analogs. More results will be provided in the next annual report.

Figures

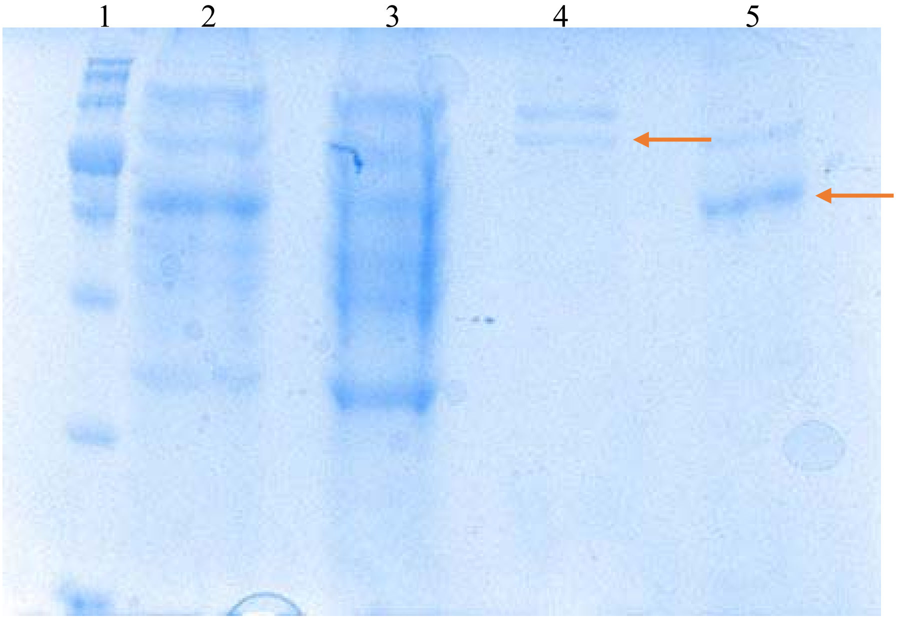

Figure 1. SDS–PAGE analysis of purified GST-TET2 (lane 4) and His-TET2 (lane 5) proteins.

	Enzyme(μg)	Activity(ng/min/mg)
1	**0**	0
2	**0.5**	10.3
3	**1**	17.4
4	**2**	42.3

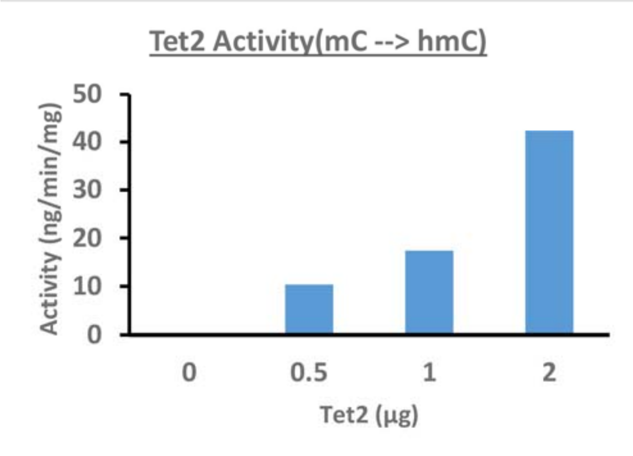

Figure 2: Colorimetric TET Activity Assay measurement using 5mC-Hydroxylase TET Activity/Inhibition Assay Kit from Epigentek.

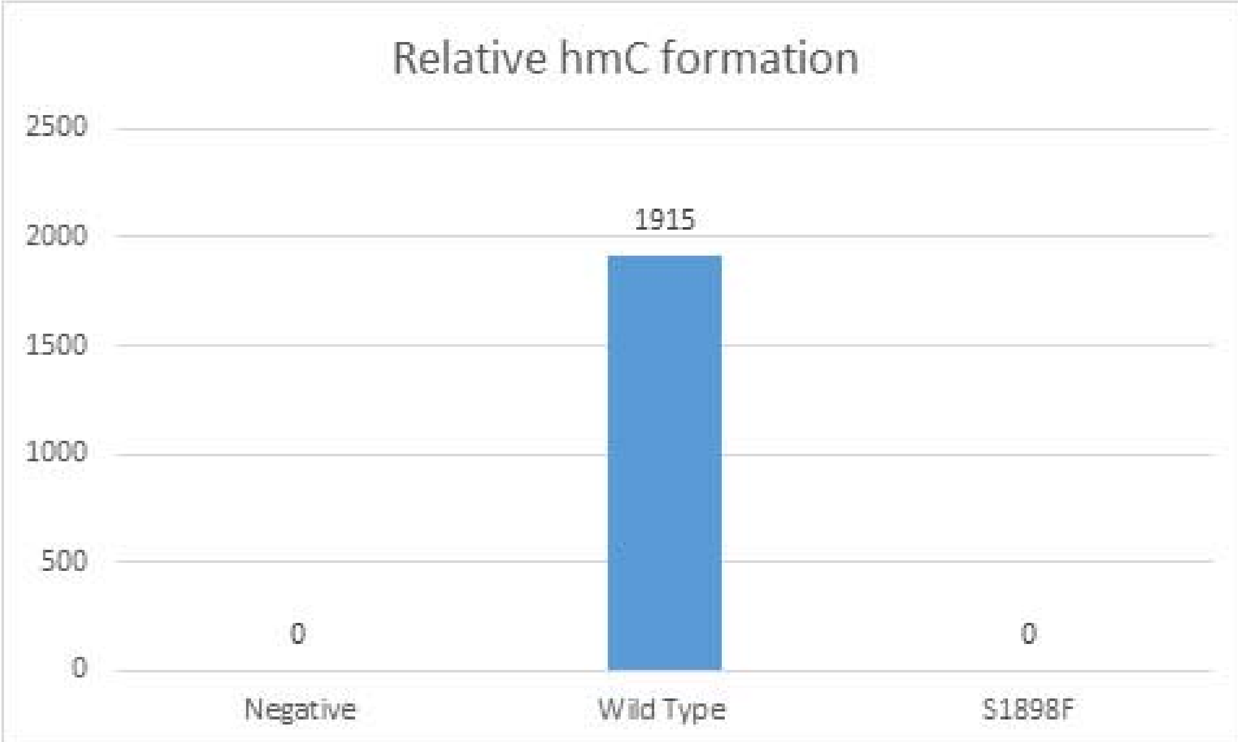

Figure 3. Liquid chromatography-mass spectrometry (LCMS) based characterization of oxidation of 5mC into 5hmC (1) in the absence of TET2 enzyme (negative), (2) in the presence of GST-wtTET2 (Wild Type), (3) in the presence of GST-S1898F-TET2 (S18989F). The enzymatic reaction samples were digested with DNAse I followed by CIAP treatment before characterization by an ABI 3200 Q-Trap mass spectrometer.

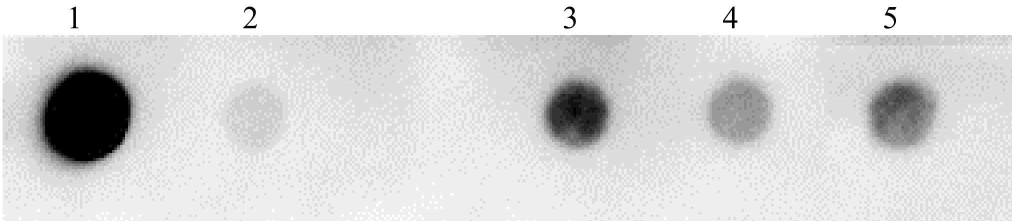

Figure 4. Dot blot analysis of GST-TET2-mediate oxidation of 5mC into 5hmC. Lane 1 shows oligonucleotide containing 5hmC used as a positive control, lane 2 shows oligonucleotide containing 5mC (substrate) used as a negative control, lane 3 shows oxidation of 5mC substrate into 5hmC by GST-wt-TET2, lane 4 shows limited oxidation of 5mC substrate into 5hmC by GST-R1896G-TET2, and lane 5 shows limited oxidation of 5mC substrate into 5hmC by GST-S1898F-TET2.

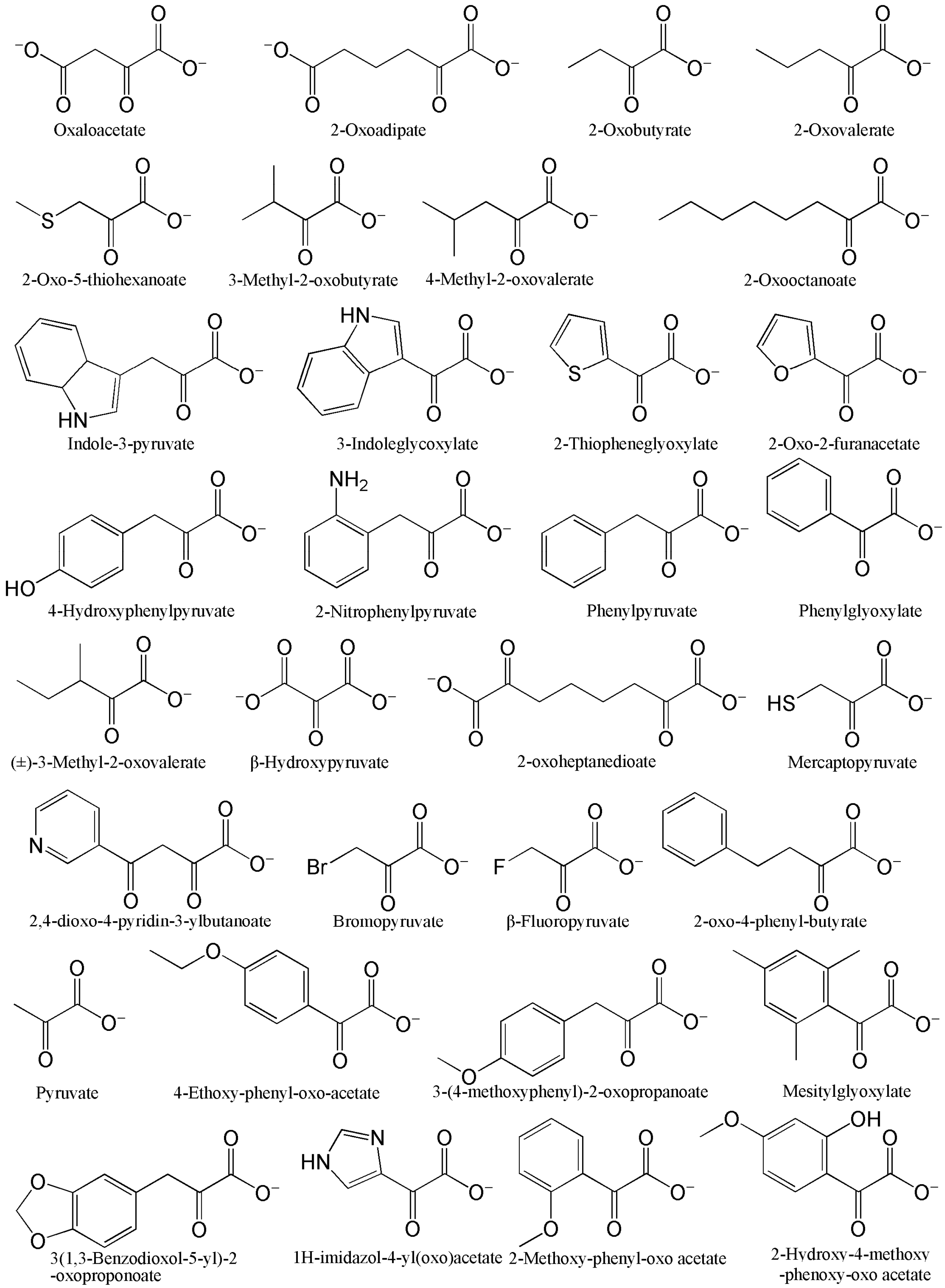

Figure 5. Structures of some 2OG analogs. These 2OG analogs will be used to rescue the activity of TET2 clinical mutants from MDS patients.

- **What opportunities for training and professional development has the project provided?**
  - This project has provided important opportunities to Dr Mridul Mukherji to attain conferences.
- **How were the results disseminated to communities of interest?**
  - Preliminary results were disseminated in a Protein Engineering conference-2015 in Chicago, IL. We are also writing few manuscripts, which would be submitted for publication soon in peer reviewed national/ international journals.
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - After some delays with the assay development, the project is moving forward. We are very excited with our initial findings showing that the activity of TET2 dioxygenase can be modified using 2-oxoglutarate analogs.
  -

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
  - For the very first time we have found that the activity of TET2 dioxygenase can be modulated using 2-oxoglutarate analogs.
- **What was the impact on other disciplines?**
  - There are many 2-oxoglutarate-depent dioxygenases like histone demethylases that regulate critical biological processes like HIF signaling, epigenetics etc. Our methods would make it possible to regulate the activity of these dioxygenases using 2-oxoglutarate analogs.
- **What was the impact on technology transfer?**

  A patent application will be filed soon.
- **What was the impact on society beyond science and technology?**
  - Nothing to Report.

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**
  - We have had issues with development of a reliable TET2 dioxygenase assay. Progress of the project has been hampered by delays in permission to use the mass spectrometry instrument with ammonium salt (please note that our LCMS method requires use of ammonium salt in the buffer for

proper separation of nucleosides). However, now we are able to use ammonium salt in the LCMS methods.

While we were waiting for the permission to use LCMS system with ammonium salt, which took over one year, we spent significant time on developing alternative TET2 assays using (i) colorimetric TET Activity Assay from Epigentek, and (ii) Dot blot. However, both these assays detect 5mhC, using an antibody, and therefore these two assays were not very reliable because they don't detect 2nd (5fmC) and 3rd (5cmC) TET2 products. Only the LCMS assay detects all three products, namely 5hmC, 5fmC, and 5cmC, of TET2 enzyme.

Please note that at this point no changes in the objective or scope have been done.

- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - After one year we were able to use our LCMS system with ammonium salt. This has allowed us to progress with the project as proposed in the application.
- **Changes that had a significant impact on expenditures**
  - Since the alternative colorimetric assay from Epigentek requires a plate reader, we received a written permission from DOD to buy a demo molecular devices plate reader costing over $19,000. Along with the purchase of the plate reader, which was not allocated in the original approved budget by DOD, I had to pay students and buy reagents in order to develop alternative assays.
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**: Nothing to Report.
- **Significant changes in use or care of human subjects:** Nothing to Report.
- **Significant changes in use or care of vertebrate animals:** Nothing to Report.
- **Significant changes in use of biohazards and/or select agents:** Nothing to Report.

6. **PRODUCTS:** Nothing to Report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**
  - *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is*

unchanged from a previous submission, provide the name only and indicate "no change."

Name:	Mridul Mukherji
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.36
Contribution to Project:	*Dr Mukherji supervises the project on day-to-day basis and ensure that the participating graduate students learn appropriate skills to conduct their daily research independently and timely manner. He participate in writing progress reports and research publications.*
Funding Support:	

Name:	Swami Prakash
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	161-14-504 (EMPLID #)
Nearest person month worked:	3
Contribution to Project:	He is working on purification and assay of TET2.
Funding Support:	

Name:	Mohit Jaiswal
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	161-29-343 (EMPLID #)
Nearest person month worked:	3
Contribution to Project:	He is working on purification and assay of TET2.
Funding Support:	

Name:	Saima Subhani
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	161-31-110 (EMPLID #)
Nearest person month worked:	3

Contribution to Project:	She works on structure function relationship (SAR) analysis, TET2 kinetics.
Funding Support:	

Name:	Subhradeep Bhar
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	162-02-782 (EMPLID #)
Nearest person month worked:	3
Contribution to Project:	He is responsible for LCMS-based TET2 assays.
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  Nothing to Report.

- **What other organizations were involved as partners?**
  - Nothing to Report.
    - **Organization Name:**
    - **Location of Organization:** *(if foreign location list country)*
    - **Partner's contribution to the projec**t *(identify one or more)*

8. **SPECIAL REPORTING REQUIREMENTS**: Nothing to Report.

9. **APPENDICES:** Nothing to Report.